Final Report

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The purpose of this research was to investigate the role of cell wall calcium in the control of plant gravitropism. The dogma, at the start of this project, was that there is a redistribution of cell wall calcium in gravitropism, and that the wall calcium directly inhibits the cell elongation. It was expected that the slower-growing side of both roots and stems would have greater amounts of wall calcium.

In order to test this dogma, we undertook five projects. The first involved measurement of the concentration of apoplastic free calcium (Ca_a) on the two sides of a horizontal maize root tip, using calcium-specific microelectrodes. To minimize the problem of root cap slime, which is high in calcium, we used decapped roots, which were allowed to regain their gravitropic sensitivity. These measurements showed that the Ca_a on the upper side declined by 50%, and the Ca_a on the lower side increased by a comparable amount within 10 minutes after root tips were placed in a horizontal position. This was the first direct evidence that a gradient of free calcium would occur during gravitropism.

The remainder of the studies made us of stems of dicots, since the cell layer that primarily controls the rate of cell elongation is the outer one, rather than an internal layer as in roots. The first study was to measure directly the amount of wall-bound calcium on the two sides of gravitropically-bending sunflower and pea stems and maize coleoptiles. These tissues were placed vertical or horizontal, then the stems were cut into sections, bisected into upper vs lower (or right vs left), and the outer layers separated from the inner tissues. In each case, we showed that there was simply no difference in amount of wall-bound or total tissue calcium on the upper vs lower sides, or as compared with vertical controls. There was no redistribution of calcium between external cell layers and internal tissues either. It was apparent that the basic idea that a redistribution of wall calcium was occurring was simply incorrect.

As a second test, we grow sunflower seedlings in the presence of varying levels of calcium. As the calcium in the root medium increased, there was a comparable increase in the amount of wall-bound calcium until a maximum, steady-state level was achieved. But regardless of the level of calcium, the amount of gravitropic curvature was the same. In addition, the growth rate of the seedlings was actually increased by the calcium up to external calcium levels of 20 mM. It is clear that there is no direct relationship between the amount of wall-bound calcium and the ability of cells to elongate or the ability of tissues to undergo gravicurvature.

A fourth project involved a direct test of the long-held idea that calcium crosslinks in cell walls act as stiffening agents, and that wall loosening might involve a breakage of these calcium

crosslinks. We took advantage of the fact that frozen-thawed cell walls can undergo wall extension when placed under tension. At pH 6, the extension is strictly viscoelastic, in that after an initial rapid extension, the extension nearly ceases. But when the solution pH is changed to 4.5, the walls become loosened, and long-term extension of the walls is obtained. If calcium-bridges are important stiffening agents, then removal of the calcium should permit the walls to extend. Since it was known that protons would displace wall-bound calcium, the possibility existed that acid was simply acting by cleaving these calcium bridges. It had previously been shown that the calcium chelator EGTA could cause walls to become loosened, but since EGTA exchanges Ca for H, it also acidifies the walls, and the extension response might have been due to the lowered wall pH rather than to the extraction of wall calcium. To test this, we made use of the calcium chelator Quin-2, which exchanges Ca for Na with the result that the apoplastic pH does not change. We showed that Quin-2 effectively removes wall-bound Ca from sunflower cell walls, with over 90% of the calcium being removed within 1 hour. But the extension of the frozen-thawed walls under tension was only slightly increased by Quin-2, even though it could be greatly increased by H^+ given after the Quin-2. These data leave no doubt that calcium crosslinks cannot be significant stiffening agents in cell walls. Thus even if there were a redistribution of wall-bound calcium during gravitropism, it could not be the cause of the unequal growth rates that occur on the two sides of the stem.

The last project of this series concerned the relationship between the amount of wall-bound calcium, the apoplastic free calcium and the wall pH. Since wall carboxyl groups bind both calcium and protons, and Ca^{2+} and H^+ compete for the carboxyl groups, the amount of apoplastic Ca_a and H^+ must be in equilibrium with the wall bound calcium. If one knows the Ca_a concentration and the amount of wall-bound calcium, one can calculate what the wall pH must have been. We took epidermal peels from these stems, froze and thawed them, and placed them in buffers of known pH and calcium concentration. We measured the initial level of wall-bound calcium, and the amount of wall-bound calcium that existed after a four-hour incubation. If at any Ca^{2+} concentration the solution pH was higher than that which must have existed in the wall in vivo, the walls will take up calcium, while if the pH were lower than in vivo, the walls would lose calcium. At every calcium concentration there is a pH at which the walls neither gained nor lost calcium. A plot of these "null-points" gave the possible combinations of wall pH and Ca_a that might have existed in the walls at the time there were harvested. Then we used Ca-sensitive microelectrodes to measure the Ca_a concentration of soybean hypocotyl walls. We showed that it is about 200 μM. From our "null-point" graph we could then calculate that the wall pH must have been about 3.2! This pH value is far lower than any we had expected. We realized that this must be the pH of the Donnan Free Space (DFS) rather than the Water Free Space (WFS) of the apoplast. The DFS pH is lower than the WFS pH because of H^+ that are trapped there to neutralize the wall carboxyl groups. The only common ion that can substitute for H^+ as a DFS cation is Ca^{2+}.

It has been known for many years that calcium ions can inhibit plant cell elongation, but the mechanism of this inhibition has never actually been determined. We conducted a study of the calcium-inhibition of soybean hypocotyl cell walls, and found that calcium ions cause a strong, but transitory inhibition of elongation. After a period which is determined by the calcium concentration, growth resumes, although often at a rate lower than the original rate. We have

interpreted this to indicate that external calcium ions are entering the DFS where they displace the H^+ to the WFS, raising the pH of the DFS and lowering it in the WFS. We also suggest that it is the pH of the DFS which determines the rate of wall loosening and thus cell elongation. With time, the cells react to a change in DFS pH, and excrete more H^+ with the result that the DFS pH becomes lowered and the growth resumes again.

These studies, then, have clearly established that the level of apoplastic bound calcium does not play any significant role in gravitropic curvature. The wall-bound calcium does not act as a wall-stiffening agent, and changes in its level does not alter the extensibility of cell walls. There is, however, a change in apoplastic free calcium, which is to be expected because the wall pH changes during gravicurvature. There is no evidence yet to indicate that the Ca_a level is important in the control of cell elongation, but this possibility must still be considered. The focus of attention concerning calcium and gravitropism needs to be shifted to cytoplasmic calcium instead.

In addition to these calcium projects, several other interesting results were obtained. The first concerns the cell layers in the root through which the gravitropic signal passes as it moves from the root cap to the elongation zone. To test this, we removed the cortex from maize roots from the tip to the elongation zone, in a pattern in which opposite quarters were removed. We then oriented the horizontal roots with the remaining cortex quarter either at top and bottom, or on the two sides. We reasoned that if the signal were moving through the cortex, we should see more curvature when the cortex was on top and bottom. However, the curvature was the same in all cases. Yang and Evans then reported that removal of a girdle of cortex would prevent roots from curving. When we tested this more carefully, we found that this was not correct. It appears that the gravitropic signal is not moving through the cortex.

These results have been published in ten peer-reviewed papers. The results have also been presented in four Plant Physiology and four ASGSB meetings.